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ORIGINAL ARTICLE

Beneficial Effects of Follistatin in Hepatic Ischemia-Reperfusion Injuries in Rats

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Abstract
Background Ischemia-reperfusion injury has been demonstrated in a variety of clinical settings. The morbidity associated with liver transplantation and major hepatic resections is partly a result of ischemia-reperfusion injury. Follistatin, an activin-binding protein, binds to activins and subsequently blocks their action. It was reported that blockade of the action of activin with administration of follistatin accelerates recovery from ischemia renal injury. This study was conducted to investigate the involvement of the activin–follistatin system in hepatic ischemia-reperfusion injury.
Methods Total hepatic ischemia for 30 min was performed followed by reperfusion in a rat model. Rats were divided into two groups: a follistatin group and a control group. Follistatin (1 μg/body), which is an activin-binding protein, was administered at the time of reperfusion.
Results Though 80% of animals survived in the follistatin group, four of five animals died in the control group within 3 days after reperfusion ($p < 0.05$). AST was significantly lower at 3 h after reperfusion in the follistatin group ($p < 0.05$). LDH was also lower at 6 h after reperfusion in the follistatin group ($p < 0.05$). Follistatin inhibited the mRNA expression of the βA subunit of activin. Moreover, the expression of IL-6, which is an inflammatory cytokine, was suppressed at 6 h after reperfusion in the follistatin group ($p < 0.05$).
Conclusions The present study demonstrated that treatment with follistatin reduced the expression of IL-6 and activin resulting in beneficial support for hepatic ischemia-reperfusion injuries.

Keywords Hepatic ischemia-reperfusion injury · Follistatin · Activin · Cytokine

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Introduction

Ischemia-reperfusion injury (I/R) has been demonstrated in a variety of clinical settings, such as in a myocardial infarction [1], stroke [2], and organ transplantation [3]. The possible consequences of injury caused by I/R include both primary organ failure and/or secondary multi-organ system failure that eventually lead to mortality [4]. Moreover, I/R injuries to the liver are of clinical importance in humans after hemorrhagic and cardiogenic shock, liver surgery, or liver transplantation [5]. Hepatic ischemia-reperfusion injury is a complex, multifactorial pathophysiologic process. In the early stages of reperfusion, endothelial cell

swelling, vasoconstriction, leukocyte entrapment, and platelet aggregation within the sinusoids result in failure of the microcirculation. Consequently, activation of nuclear factor-κB in the liver promotes proinflammatory cytokine and adhesion molecule synthesis. These reactions cause oxygen- derived free-radical production and neutrophil recruitment, further contributing to cellular injury. Many studies have been carried out to understand the underlying mechanisms and sequences in an attempt to determine an ideal treatment for the prevention of both primary and secondary tissue injuries caused by ischemia reperfusion [4].

Follistatin is an activin-binding protein. This protein stoichiometrically binds to activins and blocks their action. Follistatin is expressed on the surface of the target cells of activins by binding to the extracellular matrix. Activins are members of the transforming growth factor-β (TGF-β) supergene family [6, 7]. Among them, activin A, which is a homodimer βA subunit of activin, is an autocrine growth inhibitor produced in hepatocytes and tonically inhibits proliferation of hepatocytes [8, 9]. After partial hepatectomy, the expression of the βA subunit of activin abruptly drops [9, 10]. The expression of the βA subunit then increases gradually at 12–24 h after hepatectomy and remains elevated until the liver regeneration is terminated. The actions of activins are modified at several levels by various factors. Activins trapped by follistatin are internalized by endocytosis and subsequently degraded by proteolysis. It was reported that follistatin induces immediate deoxyribonucleic acid (DNA) synthesis in the remnant livers of 90% hepatectomized rat [11].

It was reported that blockade of the action of activin with administration of follistatin accelerates recovery from ischemia renal injury by accelerating regeneration after ischemia [12]. Activin was reported to support neuronal survival or neural differentiation in stroke injury [13]. However, there are no reports about the effect of follistatin on hepatic ischemia-reperfusion injuries. Therefore, in this study we evaluated the role of follistatin on hepatic ischemia-reperfusion injuries.

Materials and Methods

Animals

Male Wistar rats (180–220 g, Charles River Inc., Japan) were used after 7 days of acclimation to the animal room. The animals were allowed free access to water and standard laboratory chow ad libitum. They were fasted for 12 h before surgical procedure. The present study was conducted in compliance with the Division for Animal Research Resources, Institute of Health Biosciences, the University of Tokushima. The experiments and procedures were approved by the Animal Care and Use Committee of the University of Tokushima.

Experimental Operation Procedure of Ischemia-Reperfusion Model

The animals were randomly divided into two groups: a follistatin group ($n = 31$) and a control group ($n = 31$). The animals were anesthetized by inhalation with diethyl ether. A midline incision was made and the liver exposed. Total hepatic ischemia was induced by clamping the hepatoduodenal ligament. After 30 min of total hepatic ischemia, the clamp was removed to initiate hepatic reperfusion. At the indicated times (0, 3, 6, 12, and 24 h) after reperfusion, animals were killed ($n = 5$ each) for collection of serum and liver tissue.

Administration of Follistatin

After total hepatic ischemia and reperfusion, the cecum was washed out from the peritoneal cavity and the ileocolic vein was stretched. In the follistatin group, 1 mg of recombinant human follistatin, as in the previous study, dissolved in 0.5 ml of physiological saline, was infused into the portal vein via the ileocolic vein. The same volume of saline was infused in the control group [8, 14]. As previously described, a significant amount of recombinant human follistatin remained in the liver after intravenous infusion [8].

Survival Study

Six rats in the each group were used for the survival study. Rats that had lived for more than 4 days after reperfusion were considered to be survivors.

Histological Analysis

Liver tissue was fixed in 10% neutral buffered formalin, embedded in paraffin, and cut serially into 5-μm sections. The hematoxylin and eosin (H&E)-stained sections were evaluated at 200× magnification.

Biochemical Analysis

To evaluate the liver injury at each time point, the levels of serum aspartate aminotransferase (AST), serum alanine aminotransferase (ALT), and lactate dehydrogenase (LDH) were measured using the Japan Society of Clinical Chemistry standardization matching method. All measurements were performed by Shikoku Chuken, Inc. Kagawa, Japan.

RT-PCR for IL-6, IL-10, Activin βA, and Activin βC

At each time after reperfusion, the messenger ribonucleic acid (mRNA) expression levels of interleukin-6 (IL-6), IL-10, activin βA, and activin βC were evaluated by reverse transcriptase-polymerase chain reaction (RT-PCR). Total ribonucleic acid (RNA) was extracted from 30 mg of rat liver tissue using RNeasy Mini Kit (QIAGEN, Hilden, Germany). The amount of purified RNA was measured by ultraviolet (UV) spectroscopy at 260 nm, and its purity was determined by calculating the ratio of absorbance at 260/280 nm. Using 1 μg of total RNA isolated from each sample, synthesis of complementary deoxyribonucleic acid (cDNA) was carried out with M-MLV reverse transcriptase (Promega Co., Madison, WI). RT reactions were conducted in the presence of 25 pmol/μl random hexamer, RT 5 × reaction buffer, 2.5 mM dNTP Mix (GeneACT, Inc., Kurume, Japan), RNase inhibitor (Promega Co., Madison, WI), and reverse transcriptase in a final volume of 20 μl; reactions were conducted at 42°C for 60 min. PCR was carried out in the final volume of 20 μl containing 2 μl of cDNA, 1 × PCR buffer containing 20 mM of MgCl2 (Roche Applied Science, Basel, Switzerland), 2.5 mM of dNTP mix (GeneACT, Inc., Kurume, Japan), each specific primers and 1 unit of FastStart Taq DNA polymerase (Roche Applied Science, Basel, Switzerland). PCR was performed under the conditions shown in Table 1. PCR products were separated and stained in 2% agarose gels mixed ethidium bromide solution (10 mg/ml). After the electrophoresis and photographing, the data were analyzed using the public-domain ImageJ software (Version 1.34; developed at U.S. National Institutes of Health).

Statistical Analysis

All results were expressed as mean values ± standard deviations (SD). Comparisons between the two groups were performed with Student's t test, Mann–Whitney U test, or log-rank test for survival. Survival rate between groups of animals was compared using the log-rank test for comparison of survival curves. A p value of less than 0.05 was considered statistically significant.

Results

Survival of Animals

Four days after the experimental operation, the survival rate was significantly better in the follistatin group compared to the control group ($n = 6$ each) (Fig. 1). Four of six died in the control group within 12 h after reperfusion. The survival rates 1 week after reperfusion were 17% in control group and 82% in the follistatin group.

Effects of Follistatin on Hepatocellular Injury Induced by Ischemia Reperfusion

Hepatocellular injury was evaluated by measuring the liver enzymes (AST, ALT, and LDH). In the follistatin group, AST at 3 h, ALT at 3 h, and LDH at 3 h after reperfusion showed a significantly decrease in follistatin group compared to the levels in the control group (1,004 ± 320 vs. 2,689 ± 1225; $p < 0.05$, 558 ± 116 vs. 2,118 ± 1,193: $p < 0.05$, 5,730 ± 2,178 vs. 11,325 ± 3,105; $p < 0.05$) (Fig. 2). AST at 6 h and LDH at 6 h after the reperfusion tended to be lower in the follistatin group than the control group (1,231 ± 226 vs. 1,617 ± 349; $p < 0.1$, 2,472 ± 1,661 vs. 8,393 ± 5,840; $p < 0.1$). This suggests that follistatin may protect hepatocellular injury in the early phase of ischemia reperfusion.

Histological Assessment of Ischemia-Reperfusion Injury

In the control group, hepatocellular ballooning was shown in the peri-central area followed by 3, 6, and 12 h of reperfusion (Fig. 3a–c). In the follistatin group, the occurrence of hepatocellular ballooning in the peri-portal area was reduced compared to the control group followed by 3, 6, and 12 h of reperfusion (Fig. 3e–g). In the control group, hepatocellular necroses were shown in peri-central area at 3 and 6 h after reperfusion. At 12 h after reperfusion, the necrotic area was increased and shown in peri-central area (Fig. 3d). On the other hand, almost normal structures were shown in the follistatin group after 12 h of

Table 1 Sequence of synthetic oligonucleotide primers and expected fragment sizes of PCR products in the rat

Gene	Primer sequence	Size (bp)
IL-6	5′-GAG GAT ACC ACC CAC ACC AGA CCA GTA-3′	525
	5′-GGT TTG CCG AGT AGA CCT CAT AGT GAC-3′	
IL-10	5′-TGC CTT CAG TCA AGT GAA GAC-3′	346
	5′-AAA CTC ATT CAT GGC CTT GTA-3′	
Activin βA	5′-GAG AGG AGT GAA CTG TTG CT-3′	605
	5′-TAC AGC ATG GAC ATG GGT CT-3′	
Activin βC	5′-CCA TAT GAC ACC AAC CTC ACC-3′	543
	5′-GAC AAT GTT GCT GTC CCT GTC-3′	

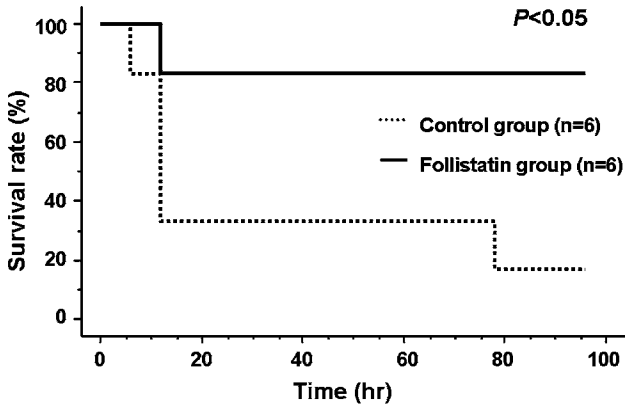

Fig. 1 Survival after ischemia reperfusion. Five of six animals in the control group died during 3 days after reperfusion, whereas survival significantly improved in the follistatin group

reperfusion (Fig. 3h). The extent of the necrosis was significantly reduced in the follistatin group at 12 h after reperfusion (Fig. 3i).

Cytokine Response

The highest expression of IL-6 was observed at 6 h after reperfusion in the control group (Fig. 4a). Administration of follistatin significantly reduced the IL-6 response at 6 h after reperfusion (0.075 ± 0.025 vs. 0.926 ± 0.336; $p < 0.05$). After 12 h of reperfusion, there was no significant difference in IL-6 between the two groups. The levels of IL-10, which is an anti-inflammatory cytokine, increased at 6 h after reperfusion in both groups, but there were no significant differences between the two groups (Fig. 4b).

Expression of Activin Subunits

Expression of the βA subunit of activin was significantly reduced at 6 h after reperfusion in the follistatin group (0.344 ± 0.204 vs. 0.841 ± 0.206; $p < 0.05$) (Fig. 5a). However, the expression of the βC subunit of activin showed no significant difference in two groups after 12 h reperfusion. The mRNA expression of the βC subunit of activin showed no significant difference due to administration of follistatin (Fig. 5b).

Discussion

Ischemia-reperfusion injury of the liver is clinically relevant in hepatic resectional surgery, live transplantation, and hemorrhagic shock [5]. In the present study, we examined the effects of follistatin on liver ischemia-reperfusion injury. The overall results demonstrate that administration of follistatin reduces the liver damage caused by ischemia

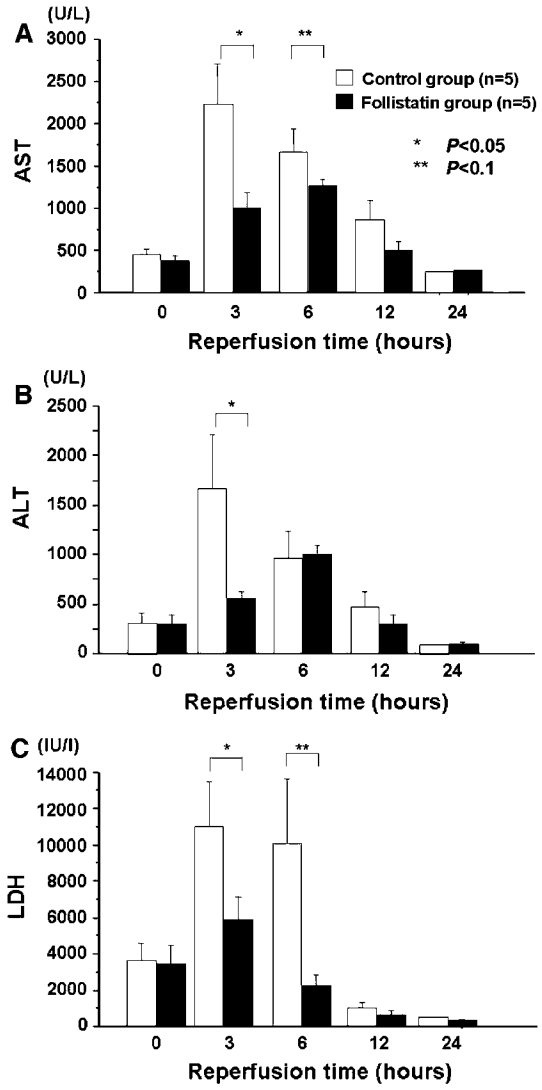

Fig. 2 Inhibition of hepatic enzyme release. Rats were given infusion of follistatin (*black bar*) or saline solution (*white bar*) at the time of reperfusion. Serum aspartate aminotransferase (AST) levels (**a**), serum alanine aminotransferase (ALT) levels (**b**), and lactate dehydrogenase (LDH) levels (**c**), were measured at the indicated time points. Values are mean ± SEM, $*p < 0.05$, $**p < 0.1$ versus saline solution

reperfusion with a resultant improvement of survival. This is the first study that examines the role of follistatin in liver ischemia-reperfusion injuries, though the number of animals included in this study was relatively small.

Activin A is an autocrine-negative regulator of DNA synthesis in hepatocytes. In addition, activin A also regulates the functions of non-parenchymal cells. For example, activin A augments tubulogenesis of sinusoidal endothelial cells, and the collagen production in hepatic stellate cells is stimulated by activin A. This is an important role of activin A because reconstruction of the hepatic sinusoid is critical for the reorganization of the liver architecture during liver

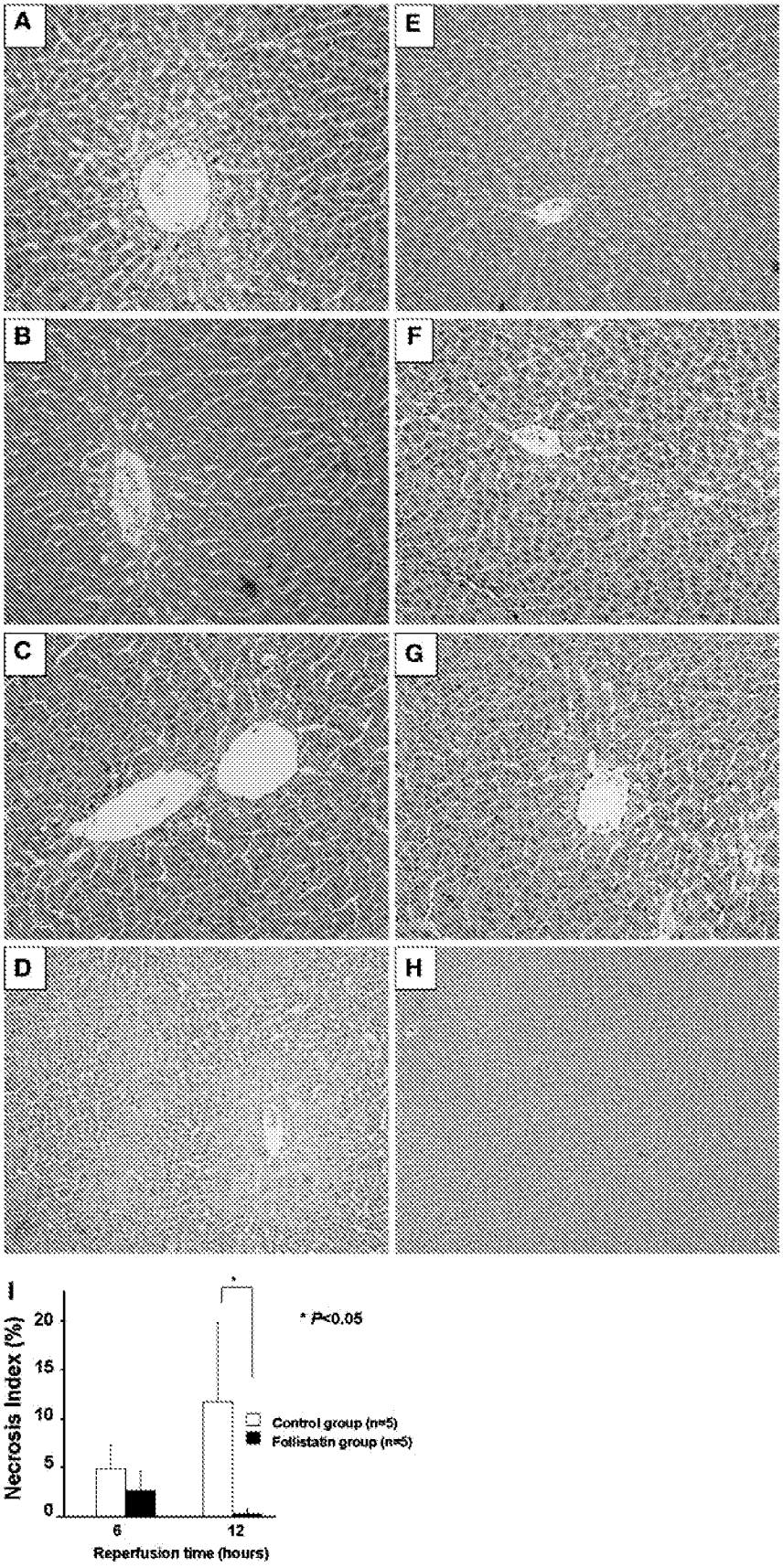

Fig. 3 Histology of the liver after ischemia reperfusion. Liver specimens were obtained and H&E staining was performed for 3, 6, 12 h reperfusion in the control group (**a–c** photographs are at 100× magnification) and in the follistatin group (**e–g** photographs are at 100× magnification). **d**, **h** H&E-stained histology for 12 h reperfusion in the control group and the follistatin group (photographs are at 200× magnification). **i** Necrosis index was calculated. Results were expressed as the ratio of the necrotic area to the whole area in high-power fields (200× magnification)

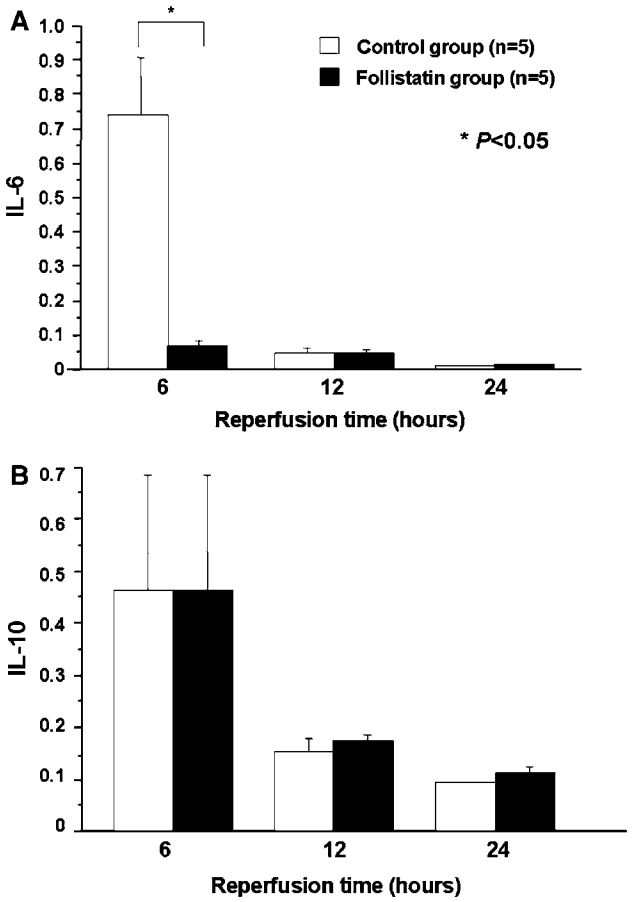

Fig. 4 Effect of follistatin to ischemia reperfusion on expressions of cytokines

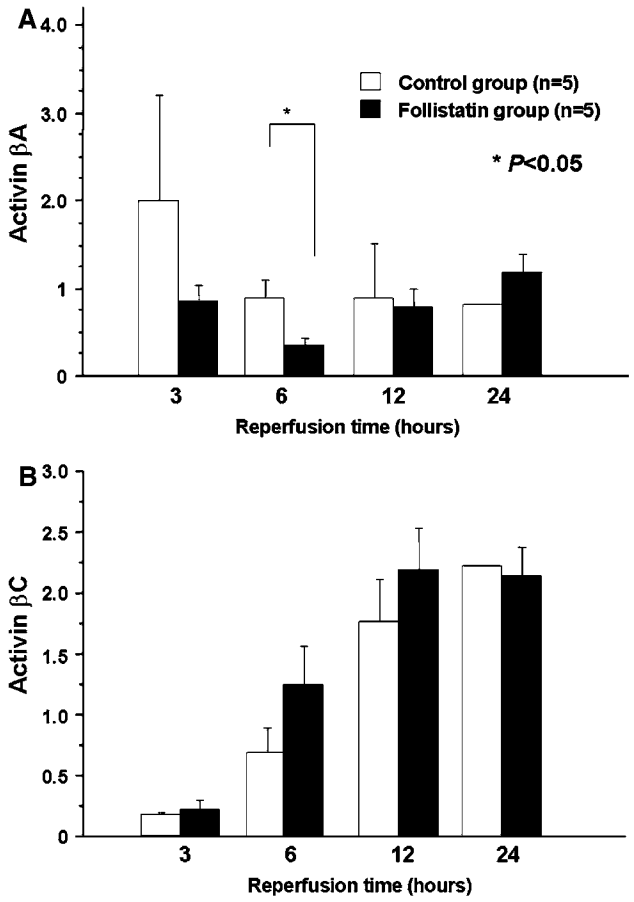

Fig. 5 mRNA changes after ischemia reperfusion. Quantitative real-time PCR reveals an increase in mRNA for **a** activin βA subunit and **b** activin βC subunit. Values are mean ± SEM, $*p < 0.05$, $**p < 0.1$ vs. saline solution

regeneration. Blockade of activin action by administration of follistatin accelerates liver regeneration after partial hepatectomy [8, 9]. In ischemia-reperfusion injury, it was reported that TGF-β_1 protects the heart from ischemia-reperfusion injury. TGF-β inhibits tumor necrosis factor-α (TNF-α) release and improve endothelium-dependent relaxation, and preserve of reactive oxygen species (ROS) generation. Moreover, TGF-β suppresses expression of Matrix metalloproteinase-9 (MMP-9) and MMP-12 in monocytes and macrophages induced by cytokines, such as IL-1β and TNF-α, results in the protection of myocardium from the adverse effects of ischemia reperfusion and improvement of cardiac function. In cerebral ischemia-reperfusion injury, it was reported that activin protects neurons from ROS induced toxicity. On the other hand, in renal ischemia reperfusion, levels of mRNA for the βA subunit of activin were up-regulated at 12 h after ischemia [5]. Maeshima et al. reported that follistatin accelerated renal regeneration and attenuated histological changes and improved renal function in renal ischemia [12].

In this study, we examined the effects of administration of follistatin on hepatic ischemia-reperfusion injury. The present study showed that the administration of follistatin down-regulated mRNA for βA subunit of activin and improved the liver function and survival rate. These results suggested that the acceleration of regeneration induced by exogenous follistatin after ischemia-reperfusion injury resulted in the beneficial effects in hepatic ischemia reperfusion.

Inflammation is also considered to be one of the most important causes of tissue injury in organs subjected to ischemia. Recent studies have suggested a novel role for activin in inflammation and repair processes in various organs [14–17]. For example, increased expression of activin was observed in various types of inflammatory processes, including cutaneous wound repair and inflammatory arthropathies. The level of activin expression correlated with the degree of inflammation in inflammatory bowel disease. Strong expression of activin A was induced in vitro by proinflammatory cytokines such as interleukin-1 and tumor necrosis factor-α (TNF-α), which are known to be released from macrophages and stromal cells at the sites of tissue injury and inflammation [18]. Therefore,

proinflammatory cytokines are possible inducers of activin expression in this model. Regarding the action mechanism of activin, the release of activin into the circulation precedes the release of proinflammatory cytokines after lipopolysaccharide (LPS) treatment, suggesting a proinflammatory action of activin [16]. Activin A is released into the circulation at very acute phase in response to LPS. Activin A is capable of stimulating the production of the pro-inflammatory cytokines TNF-α and IL-1β. It was reported that administration of follistatin decreased releases of TNF-α and IL-1β by blocking the action of the activin [19]. In contrast, activin A produces anti-inflammatory effects by blocking the action of IL-6. It was reported that activin A significantly stimulated production of IL-6 at lower concentration, while at higher concentration production of IL-6 was significantly inhibited in human amnion calls [20]. On the other hand, IL-6 level was also significantly lower with follistatin treated group in colitis model of mouse [21]. In this study, administration of rh-follistatin reduced expression of IL-6 after ischemia reperfusion. This suggests that rh-follistatin may exert an anti-inflammatory action by blocking activin in hepatic ischemia reperfusion.

Finally, our results suggest a novel and important role of follistatin in the repair process of the hepatic ischemia-reperfusion injury. It is reported that it is acceptable to give 1 μg ~ 1 mg/kg of follistatin to human (patent; JP, 4483276, B). However, the safety and toxicity of this drug for human use has not been revealed completely. So, we need clinical trials to determine the safety, proper dosage, efficacy, and adverse reactions of this drug. Follistatin has therapeutic potential for the prevention and treatment of hepatic damage leading to acute liver failure.

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